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Selection of Xenobiotic-Degrading Microorganisms in a Biphasic Aqueous-Organic System

MIGUEL ASCON-CABRERA and JEAN-MICHEL LEBEAULT*

Division des Procédés Biotechnologiques, Département Génie Chimique, Centre de Recherches de Royallieu, B.P. 649, Université de Technologie de Compiègne, 60206 Compiègne Cédex, France

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Microbial selection on mixtures of chlorinated and nonchlorinated compounds that are poorly soluble in water and/or toxic to growing microbial cells was examined in both biphasic aqueous-organic and monophasic aqueous systems. A biphasic system in which silicone oil was used as the organic phase permitted the acceleration of acclimation, leading to rapid selection and to an increase in xenobiotic compound degradation. In contrast, acclimation, selection, and degradation were very slow in the monophasic aqueous system. The variation in microbial growth rate with the degree of dispersion (i.e., dispersion at different silicone oil concentrations and agitation rates), and cell adhesion to the silicone oil indicate that the performance of the biphasic aqueous-organic system is dependent on the interfacial area between the two phases and that microbial activity is important at this interface. Therefore, the biphasic water-silicone oil system could be used for microbial selection in the presence of xenobiotic compounds that are toxic and have low water solubility.

The selection of microorganisms able to grow on xenobiotic compounds is the first problem to solve in the biodegradation process. The isolation of adapted microorganisms with the required degradative capacities is usually preceded by an acclimation period; acclimation is generally accomplished by using classic batch and continuous enrichment techniques (8, 10).

Acclimation of microorganisms on a xenobiotic compound, which can be defined either as a decrease in the lag period (18) or functionally as an increase in the degradation rate (1), is the result of several mechanisms, including the induction or derepression of enzymes, mutation or genetic exchange, multiplication of the initially small populations of degrading organisms, an insufficient supply of inorganic nutrients, preferential utilization of other organic compounds before the chemical of interest, adaptation to the toxins or inhibitors present, and predation by protozoa (1, 18, 32).

While many genera of microorganisms that use xenobiotic compounds as growth substrates have been isolated, attempts to isolate strains in the presence of a specific xenobiotic compound, even from an enrichment culture, are not always successful. Three main reasons have been found. First, some compounds may be partly or completely degraded by cooxidation or cometabolism that requires an additional substrate (3, 14). Second, the compound in question may be degraded only by a microbial consortium, with no single organism possessing all of the required characteristics (3, 14, 15). And third, the degradation process may require interfaces and/or gradients (5, 7, 30). This last factor is applicable mainly to toxic and sparingly soluble substances in aqueous environments.

Microbial selection on xenobiotic compounds that are poorly soluble in water and/or toxic to growing microbial cells often requires extremely long acclimation periods (several months) (11, 27, 29). This becomes, in itself, a limiting step in the degradation process. Therefore, there is a need to improve the selection process. Medium engineering (16), defined as the modification and/or optimization of a microenvironment by introducing additives (e.g., organic solvents), can improve the selection process with these compounds. When a toxic substrate with low aqueous solubility is mixed with an aqueous medium, the cells suspended in the aqueous phase are in contact with low concentrations of dissolved substrate, while the cells adhering to insoluble droplets are in contact with higher concentrations of the insoluble substrate. In both cases the cells exhibit low metabolic activity because of low aqueous substrate concentration or because of toxicity at the liquid-liquid interface. In contrast, if the substrate is dissolved in an organic solvent, its concentration decreases in the reactor and may drop below toxic concentrations, resulting in greater cellular activity (7, 19, 23, 25). In the biphasic aqueous-organic system, the substrate diffuses from the organic phase to the aqueous phase, which contains mineral salts. Microorganisms carry out substrate conversion in the interfacial area and/or aqueous phase, while the metabolites that have low aqueous solubility can be extracted by the organic phase (5, 12, 19). Thus, in this system it is possible to avoid substrate or product inhibition. The biphasic aqueous-organic system is extensively used for microbial and enzymatic bioconversions of poorly water-soluble substrates in a single-step reaction (5, 7, 12, 16, 19). However, it has been demonstrated that this system can be used for complex oxidation reactions and complete mineralization of substrates that are only sparingly soluble in aqueous environments (9, 20, 22, 33). Several water-miscible, water-immiscible, and hydrophobic solvents can be used as the organic phase (7, 16, 19). However, it is more suitable to use hydrophobic solvents because their low polarity leads to higher activity and stability of microbial cells (12, 16, 17, 19, 20, 25). Prokop et al. (23) have studied the *n*-hexadecane degradation of a water-dewaxed gas oil system, and Wodzinski and Larocca (33) have used the water-heptamethylnonane system for degradation of naphthalene, while Efroymeon and Alexander (9) have used the same system for studying naphthalene and *n*-hexadecane degradation. Penaud (22), using the water-silicone oil system, has studied toluene degradation.

Silicone oils have been widely used in industrial applica-

* Corresponding author.

TABLE 1. Values for octanol/water partition coefficients, chemical solubilities in water, and toxicity parameters for the xenobiotic compounds used

Substrate	K_{ow}[a]	Solubility in water (log mM)	Actual solubility in aqueous phase (mg/liter)[b,c]	IC_{50} (mg/liter) for[d]:	
				Mixed cultures[c,e]	Aerobic heterotrophs[f]
1,2-Dichlorobenzene	3.42	−0.06[g]	0.240	850	910
1,2,3-Trichlorobenzene	4.09	−0.89[g]	0.051	890	NR[h]
1,2,4-Trichlorobenzene	3.98	−0.76[g]	0.066	970	1,700
Ethyl butyrate	2.05	1.75[c]	5.51	1,200	NR
2-Ethylbutyraldehyde	2.13	1.68[c]	4.60	1,500	NR
Butyraldehyde	1.18	S[i]	38.75	1,800	NR
Ethyl acetate	0.75	S	102.50	2,100	NR
Ethanol	−0.32	S	490.00	2,350	24,000

[a] K_{ow}, octanol/water partition coefficient. Calculated by using the fragment constants of Rekker and de Kort (24).
[b] Measured in a biphasic system containing 20% silicone oil as the organic phase.
[c] Determined experimentally in this study.
[d] IC_{50}, concentration that inhibited the culture by 50%.
[e] Consortia selected on medium containing chlorinated and nonchlorinated compounds.
[f] Data from reference 4.
[g] Data from reference 21.
[h] NR, not reported.
[i] S, water soluble according to the Merck catalog.

tions. Because of their hydrophobic properties, their high levels of thermal stability, their resistance to photooxidation (6), and their biodegradation and biodegradation inhibition characteristics (31), which are all extremely advantageous factors, we selected a silicone oil as the organic phase for our experiments.

In this paper we describe the performance of a biphasic water-silicone oil system for selection of microorganisms in the presence of the following two industrial mixtures: (i) 1,2-dichlorobenzene and 1,2,3- and 1,2,4-trichlorobenzenes (low water solubility), and (ii) ethyl butyrate, 2-ethylbutyraldehyde (low water solubility), butyraldehyde, ethyl acetate, and ethanol. Factors responsible for improvement of xenobiotic compound degradation in this system and possible applications in biodegradation processes are discussed.

MATERIALS AND METHODS

Biphasic system. The biphasic aqueous-organic system was obtained by using different fractions of silicone oil 47V20 (Rhône Poulenc Co., Neuilly-sur-Seine, France) as the organic phase. The properties of silicone oil 47V20, which is available at a very high level of purity, are as follows (as determined at 25°C): fluid type, polydimethylsiloxane; molecular weight, 2,000; viscosity, 20 centistokes; density, 0.95; surface tension, 20.6 dynes/cm; dielectric constant, 2.72. This phase contained different concentrations of chlorinated or nonchlorinated mixed compounds, including 1,2-dichlorobenzene, 1,2,3- and 1,2,4-trichlorobenzenes, ethyl butyrate, 2-ethylbutyraldehyde, butyraldehyde, ethyl acetate, and ethanol, whose aqueous solubilities and toxicities are described in Table 1. A mineral salts medium (MSM) was used as the aqueous phase. The MSM contained (per liter) 775 mg of K_2HPO_4, 350 mg of KH_2PO_4, 100 mg of $(NH_4)_2SO_4 \cdot 7H_2O$, 40 mg of $CaCl_2$, 1 mg of $FeSO_4 \cdot 7H_2O$, 1 mg of $MnSO_4 \cdot H_2O$, and 0.21 mg of Na_2MoO_4. The agitation rates were 120 rpm for 250-ml baffled flasks and 200 to 800 rpm for the 2-liter reactor.

Enrichment and isolation of mixed cultures. Mixed cultures were isolated from an activated sludge collected from an industrial plant at Compiègne, France. For selection of the desired microbial consortia, standard batch enrichment culture techniques were performed with both the biphasic water-silicone oil system described above and an aqueous monophasic system. The chlorinated and nonchlorinated compounds were used as sole carbon and energy sources. Portions of the sludge sample were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of the biphasic medium, which consisted of 80 ml of MSM and 20 ml of organic phase containing the mixed substrates (0.5 g of each substrate per liter). The preparation was incubated at room temperature (18 to 22°C) and stirred at 120 rpm. When growth was observed, as determined by an increase in turbidity and a decrease in pH, 5 ml of the suspension was transferred to a new flask containing the same mixed-substrate concentrations. Samples of the enrichment culture were spread on MSM agar plates containing 100 to 200 mg of a xenobiotic compound per liter as the sole carbon source. After 8 days on this medium, 1- to 2-mm colonies appeared. The predominant colony types were picked and isolated for later taxonomic characterization. After characterization, and in order to quickly obtain cells, the isolates were routinely grown at 25°C on nutrient and Sabouraud agar plates (Biokar Laboratories, Prolabo-Rhône Poulenc Co., Paris, France) containing glucose as the sole carbon source.

Characterization of microorganisms. Isolates were streaked repeatedly on MSM agar plates containing the xenobiotic compounds to ensure purity. Characterization was based on conventional tests, such as the API 20E, API 20B, API 20NE, and API 20C-AUX tests (API System S.A., Marcy-l'Etoile, France) and the Yeast System Pasteur (Diagnostic Pasteur, Marnes-la-Coquette, France). Both the isolated and mixed cultures were maintained on nutrient MSM agar plates containing low concentrations of xenobiotic compounds (25 to 50 mg/liter). The preparations were stored in 20% glycerol at −80°C.

Degradation kinetics. Batch culture experiments were conducted in a 2-liter reactor (LSL Biolafitte S.A., Saint Germain en Laye, France) containing 1 liter of the biphasic medium described above (MSM and different concentrations of silicone oil containing various concentrations of single or mixed substrates) and inoculated with monocultures or mixed cultures. Cultures were incubated at 25°C, at various agitation rates, and at pH 4.5 (pH was controlled by addition of 10% NaOH). Samples of the cultures were removed periodically to be analyzed as described below.

The performance of a biphasic aqueous-organic system may be described by the following mass balance equation:

$$\frac{dS_a}{dt} = K_L a\,(S_i - S_a) - q_s X \qquad (1)$$

where K_L is the liquid-liquid substrate mass transfer coefficient, a is the specific interfacial area, S_i and S_a are the substrate concentrations in the interface and aqueous phase, respectively, X is the biomass concentration, and q_s is μ/Y (the specific substrate uptake rate, where μ is the specific growth rate and Y is the biomass yield coefficient). Equation 1 is used when the kinetic reaction takes place uniformly in the aqueous phase. However, the reaction can occur exclusively at the liquid-liquid interface or be unevenly distributed between the interface and the aqueous phase (19, 23).

We assumed that microbial growth occurs both at the liquid-liquid interface and in the aqueous phase. Therefore, we did not evaluate the substrate transport between the two liquid phases but evaluated only the substrate used for microbial growth. The growth kinetics in the total multiphase liquor can be described by the following Monod-Haldane equation:

$$\mu = \frac{\mu_{max}\,S}{K_s + S + \dfrac{S^2}{K_i}} \qquad (2)$$

where μ_{max} is the maximum specific growth rate, K_s is the saturation constant, K_i is the inhibition constant, and S is the total substrate concentration in the reactor. The specific growth rates were obtained from the biomass dry weight, optical density, and NaOH consumption data monitored during batch culture in biphasic systems. Kinetic parameters were estimated by using linear and nonlinear regression analyses.

Analytical methods. A technique described by Neufeld et al. (20) was modified slightly to provide an estimate of the ratio of cells adhering to the silicone oil phase to free cells in the aqueous phase. The cell broth was centrifuged at 12,000 × g for 10 min at 4°C, and the resulting pellet was washed two times in distilled water. The supernatant containing silicone oil and adhering cells was mixed with an equal volume of a solvent mixture (ethanol-acetone-chloroform, 10:10:2, vol/vol), the resulting preparation was mixed for 15 min and centrifuged, and the resulting pellet was washed three times in distilled water. The optical densities of the pellets or mixed pellets were determined by reading the A_{540} values of 5-ml samples with a WTW Mikroprozessor model MPM 1500 photometer. The dry weight of microorganisms was determined by direct weighing of the biomass after drying at 100°C for 24 h. Absorbance readings were converted to dry weights by using a linear correlation (r = 0.99; logarithmic plots).

Concentrations of the chlorinated compounds were determined by using a Intersmat model IGC 121 gas chromatograph equipped with a flame ionization detector and a 100/120 Chromosorb W-AW Alltech AT column. Growth in the presence of chlorinated compounds was measured by chloride release with a chloride-specific electrode and a reference electrode (Microprossesin pMX 2000/ION; Wissenschaftlich-Technische Werkstatten GmbH, Weilheim, Germany), using an HCl solution as the standard. The concentrations of ethanol and ethyl acetate were determined enzymatically by the UV method (biochemical analysis)

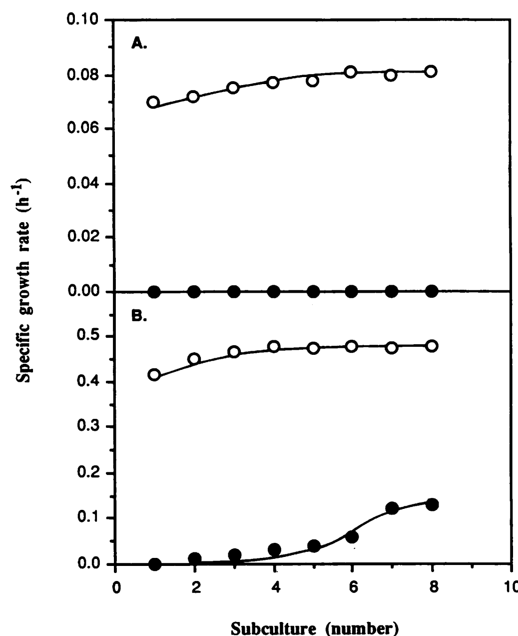

FIG. 1. Specific growth rates of initial consortia (inoculum) enriched on xenobiotic compounds as a function of subculture number in biphasic (○) and monophasic (●) systems. (A) Chlorinated compounds. (B) Nonchlorinated compounds. Preparations were subcultured every 3 and 5 days for nonchlorinated and chlorinated compounds, respectively, before they stabilized. The mixed-substrate concentrations were 1.5 and 2.5 g/liter for chlorinated and nonchlorinated compounds, respectively (0.5 g of each substrate per liter).

(Boehringer Mannheim). The concentrations of the other nonchlorinated substrates were determined by using an 80/120 Carbopack B–3% SP-1500 Supelco column.

Statistical analysis. Means, standard deviations, regressions, and analysis of variance data were determined by using StatView 512+ software (4a) and a Macintosh LC microcomputer (Apple Computer, Les Ulis, France). In general, each experiment was performed four or six times for each set of test conditions.

RESULTS

Acclimation and enrichment. With the above-described enrichment method, degradation of chlorinated and nonchlorinated mixtures was observed after about 1 week. Figure 1A shows that there was an acclimation period of about 30 days (six subcultures) before a stable consortium growth rate on medium containing mixed chlorinated benzenes was reached. During this period the specific growth rate increased from 0.070 to 0.081 h^{-1}. However, no growth was observed in the monophasic aqueous system. An acclimation period of about 20 days (four subcultures) on medium containing the nonchlorinated compounds was observed before the stable consortium growth rate was reached (Fig. 1B). During this period the growth rate increased from 0.41 to 0.48 h^{-1}, while very little growth was observed in the monophasic aqueous system. Figure 2 shows the difference between the first and last enrichment cultures, in which both consortium growth and mixed-substrate consumption were quantified. Moreover, no uptake of silicone oil during the enrichment subcultures was recorded. The growth rates of

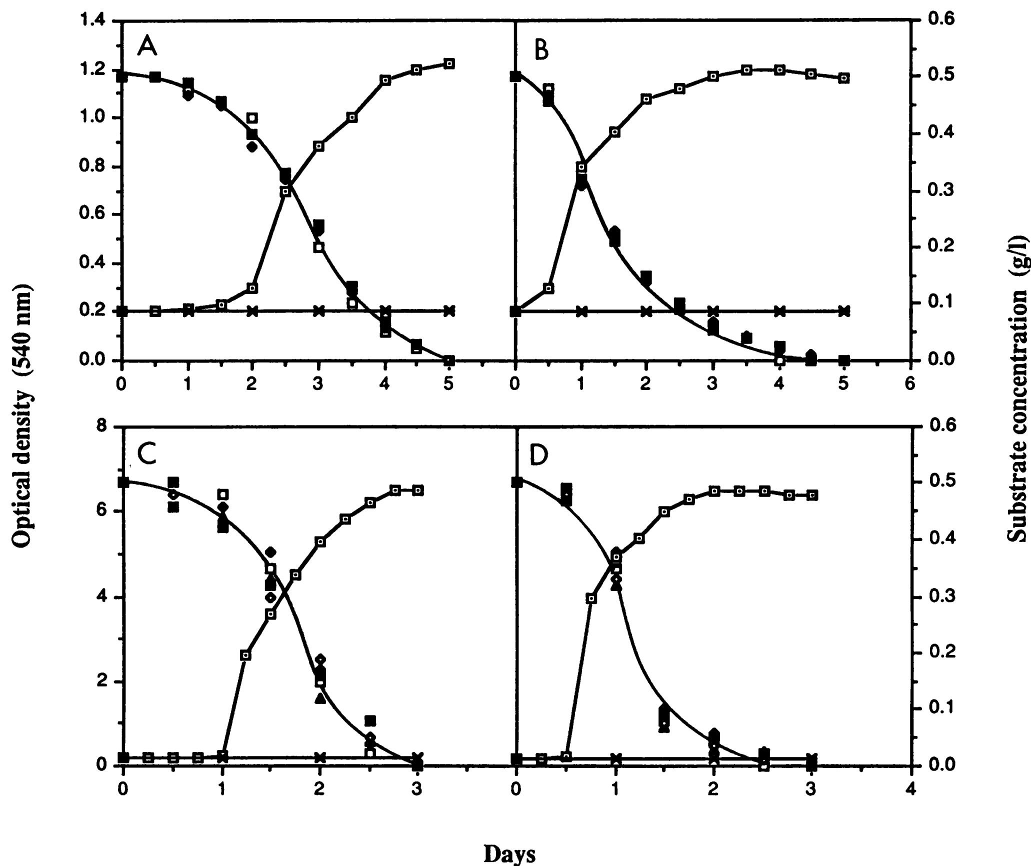

FIG. 2. Typical growth curves of initial consortia (inoculum) in a biphasic water-silicone oil system, expressed as optical density (□). (A and B) Chlorinated compounds. Symbols: □, 1,2-dichlorobenzene; ◆, 1,2,3-trichlorobenzene; ■, 1,2,4-trichlorobenzene; ×, control. (C and D) Nonchlorinated compounds. Symbols: □, ethyl butyrate; ◆, 2-ethylbutyraldehyde; ▲, butyraldehyde; ◇, ethyl acetate; ■, ethanol; ×, control. (A and C) First enrichment subculture (with nonacclimated consortia). (B and D) Last enrichment subculture (with acclimated consortia). No growth was observed in parallel control systems (i.e., cultures in biphasic water-silicone oil systems without xenobiotic compounds).

the first and last subcultures were significantly different ($P < 0.05$); however, no such significant difference existed ($P > 0.05$) between the stable specific growth rates. These data agreed well with the data presented in Fig. 3, which shows the decrease in the lag phase during the acclimation period. The lag length of the phase decreased from 35 to 10 h after 30 days of acclimation on the mixed chlorobenzenes and from 18 to 8 h after 20 days of acclimation on the mixture of nonchlorinated compounds. The differences between the lag phase values of the first and last cultures were highly significant ($P < 0.05$), as were the differences between the values obtained in biphasic and monophasic enrichment cultures on nonchlorinated compounds. Stable specific growth rates of 0.081 and 0.48 h^{-1} after lag periods of 10 and 8 h for the chlorinated and nonchlorinated compounds, respectively, were reached in biphasic water-silicone oil enrichment cultures.

Isolation of mixed cultures. At the end of the acclimation period, when the microorganisms had reached the maximal level of adaptation, samples were spread on MSM agar plates containing the xenobiotic compounds for the strains to be isolated. Mixed culture MC1, growing on chlorobenzenes, was composed of three bacterial strains, which were identified as *Pseudomonas putida*, *Pseudomonas* sp. strain Ps1, and *Alcaligenes* sp. Mixed culture MC2-b, growing on nonchlorinated compounds in the biphasic system, was composed of two bacterial strains and two yeast strains, which were identified as *Micrococcus* sp., *Pseudomonas* sp. strain Ps2, *Candida* sp., and *Trichosporon* sp. Mixed culture MC2-m, selected in the monophasic aqueous system with nonchlorinated compounds, was composed of the same two yeast strains isolated in the biphasic system and one bacterial strain identified as *Acetobacterium* sp. The characteristics used for classification of the isolates are not shown. However, the ability of each strain to grow on each compound as a sole carbon and energy source in the biphasic water-silicone oil system is shown in Tables 2 and 3.

Effect of degree of dispersion on growth kinetics. To study the effects of degree of dispersion on microbial growth, cultures of *Candida* sp. on 2-ethylbutyraldehyde were incubated in a 2-liter baffled reactor either at different agitation rates in the presence of 20% silicone oil or in the presence of different silicone oil concentrations at a fixed agitation rate of 500 rpm. In all of the cultures the initial concentration of dissolved oxygen was around 65% of oxygen saturation, and the dissolved oxygen concentration never dropped below 15 to 25% of oxygen saturation at the end of fermentation. Figure 4 shows the variation in specific growth rate as a function of different silicone oil concentrations and agitation rates. The growth rate was minimal in the absence of silicone

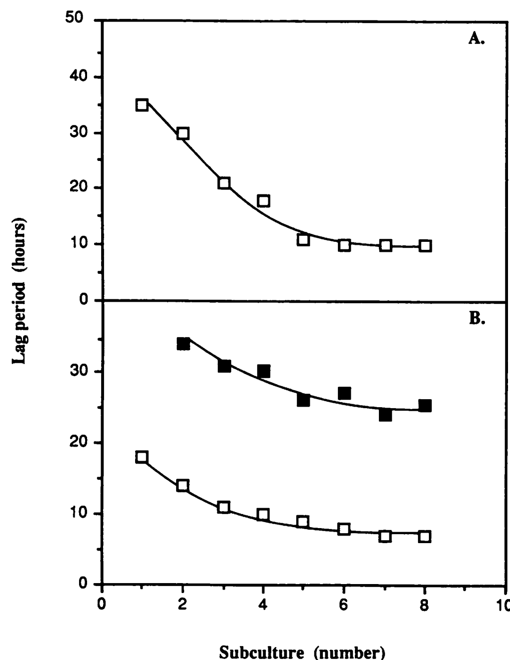

FIG. 3. Lag phases of initial consortia (inoculum) enriched on chlorinated and nonchlorinated compounds as a function of number of subcultures in biphasic (□) and monophasic (■) systems. (A) Chlorinated compounds. (B) Nonchlorinated compounds. Preparations were subcultured every 3 and 5 days for the nonchlorinated and chlorinated compounds, respectively, before they stabilized. The total mixed-substrate concentrations were 1.5 and 2.5 g/liter for the chlorinated and nonchlorinated compounds, respectively (0.5 g of each substrate per liter).

oil and at the lower agitation rate (μ, 0.09 and 0.11 h^{-1}, respectively) and maximal (μ, 0.20 h^{-1}) in the presence of 20 to 40% silicone oil and at agitation rates of 400 to 700 rpm. At higher silicone oil concentrations and agitation rates growth decreased. Therefore, these results show that optimal microbial growth occurred in a biphasic system with 20% silicone oil and an agitation rate of 500 rpm.

Performance of the biphasic system. To compare the efficiency of the biphasic organic-aqueous system with that of a monophasic aqueous system in the xenobiotic compound degradation process, batch cultures of isolated consortium MC2-b were incubated in the presence of different concentrations of nonchlorinated mixed substrates in both systems. Figure 5 shows that the performance of the biphasic system was superior to that of the monophasic system with respect to xenobiotic compound degradation. Although in both systems ca. 2.5 g/liter was the optimal concentration of the xenobiotic compounds, this concentration had different effects on microbial activity in the two systems. The specific growth rate was about two times higher in the biphasic system than in monophasic system. The maximum specific growth rate was 0.48 h^{-1} in biphasic system and 0.27 h^{-1} in the monophasic system. Likewise, the substrate inhibition effect was lower in the biphasic culture (K_i, 16 g/liter) than in the monophasic culture (K_i, 8.5 g/liter). However, similar saturation constants (K_s, 0.6 g/liter) were observed in both systems. A statistical analysis of these data showed that the biphasic system data differ significantly ($P < 0.05$) from the monophasic system data. Thus, it was established that the biphasic system is more efficient than the monophasic system during the xenobiotic compound degradation process.

Adherence capacity of cells. Although cell surface hydrophobicity was not measured, microorganisms such as *Micrococcus* sp., *Candida* sp., and *Trichosporon* sp. cultivated in the biphasic system exhibited strong adherence to silicone oil. Figure 6 shows the partitioning of the *Trichosporon* sp. cells between the water and silicone oil phases during ethyl butyrate degradation. During growth experiments, it was observed that cells adhered to silicone oil after the agitator was stopped. After 24 h of culture, 55% of the biomass was found free in the aqueous phase, while 45% of the biomass was observed bound to the silicone oil phase. These results and the microscopic observations (unpublished data) carried out during fermentation indicate that cell growth could occur both in the aqueous phase and at the water-silicone oil interface.

DISCUSSION

Using the biphasic water-silicone oil system, we selected two stable consortia from enrichment cultures on two media containing mixtures of different compounds in a relatively short time. Acclimation periods of about 30 days for chlorobenzenes and 20 days for nonchlorinated compounds were observed. We do not have data from other authors concerning microbial selection on the nonchlorinated compounds used. However, our results are different from those obtained by Haigler et al. (11), Schara et al. (27), and Spain and Nishino (29), who selected pure cultures of microorganisms on dichlorobenzenes after 10 and 14 months of acclimation. The differences among these results may be explained by the techniques used for selection and by the different microorganism sources used as inocula. These authors utilized sewage, soil, and water samples as inocula and a classic

TABLE 2. Growth rates of monocultures and a mixed culture on single and mixed chlorinated substrates in the biphasic water-silicone oil system[a]

Organism(s)[b]	Specific growth rate (h^{-1}) on:			
	1,2-Dichlorobenzene	1,2,3-Trichlorobenzene	1,2,4-Trichlorobenzene	Mixed substrates
P. putida	0.040 ± 0.001[c]	0.030 ± 0.003	0.040 ± 0.001	0.035 ± 0.003
Pseudomonas sp. strain Ps1	0.035 ± 0.001	0.046 ± 0.002	0.040 ± 0.001	0.045 ± 0.001
Alcaligenes sp.	0.040 ± 0.001	NG[d]	0.042 ± 0.001	0.040 ± 0.001
Mixed culture MC1	0.073 ± 0.002	0.080 ± 0.001	0.078 ± 0.001	0.081 ± 0.001

[a] Growth rates were determined at 25°C, pH 4.5, and 500 rpm in the presence of 20% silicone oil. The initial substrate concentrations were 1 g/liter for single substrates and 0.5 g/liter for each compound in the mixed-substrate preparation. A 2-liter reactor containing 1 liter of biphasic medium was used.
[b] Organisms selected only in the biphasic water-silicone oil enrichment culture.
[c] Mean ± standard deviation for three experiments.
[d] NG, no growth occurred on the xenobiotic compound.

TABLE 3. Growth rates of monocultures and mixed cultures on single and mixed nonchlorinated substrates in the biphasic water-silicone oil system[a]

Organism(s)	Specific growth rate (h^{-1}) on:					
	Ethyl butyrate	2-Ethyl-butyraldehyde	Butyraldehyde	Ethyl acetate	Ethanol	Mixed substrates
Micrococcus sp.[b]	0.18 ± 0.008[c]	0.16 ± 0.009	NG[d]	0.24 ± 0.013	0.22 ± 0.008	0.18 ± 0.009
Pseudomonas sp. strain PS2[b]	NG	NG	0.17 ± 0.008	0.15 ± 0.007	0.15 ± 0.006	0.14 ± 0.006
Candida sp.[e]	0.14 ± 0.005	0.15 ± 0.006	NG	0.20 ± 0.012	0.15 ± 0.007	0.16 ± 0.008
Trichosporon sp.[e]	0.14 ± 0.006	0.13 ± 0.005	0.09 ± 0.003	0.10 ± 0.004	0.13 ± 0.004	0.12 ± 0.004
Acetobacterium sp.[f]	0.15 ± 0.009	NG	NG	0.18 ± 0.011	0.20 ± 0.008	0.35 ± 0.013
Mixed culture MC2-b[g]	0.30 ± 0.024	0.28 ± 0.018	0.18 ± 0.013	0.41 ± 0.016	0.39 ± 0.012	0.40 ± 0.018
Mixed culture MC2-m[h]	0.25 ± 0.013	0.20 ± 0.012	0.15 ± 0.009	0.31 ± 0.015	0.30 ± 0.014	0.32 ± 0.016

[a] Growth rates were determined at 25°C, pH 4.5, and 500 rpm in the presence of 20% silicone oil. The initial substrate concentrations were 1 g/liter for single substrates and 0.5 g/liter for each compound in the mixed-substrate preparation. A 2-liter reactor containing 1 liter of biphasic medium was used.
[b] Organism selected only in the biphasic water-silicone oil enrichment culture.
[c] Mean ± standard deviation for three experiments.
[d] NG, no growth occurred on the xenobiotic compound.
[e] Organism selected in both the biphasic and monophasic culture systems.
[f] Organism selected only in the monophasic aqueous enrichment culture.
[g] Mixed culture selected in the biphasic water-silicone oil enrichment culture.
[h] Mixed culture selected in the monophasic aqueous enrichment culture.

enrichment technique with a monophasic aqueous system, as well as low substrate concentrations (0.3 to 5 mg/liter), which could have been below the threshold concentration for adaptation response (18). In contrast, we used an activated sludge as the inoculum, and our screening method was based on selection pressure imposed by the following two factors: (i) modification of the microenvironment by the presence of an organic phase (7, 16, 28), which avoided substrate inhibition phenomena and favored the metabolic activity of the microbial consortium; and (ii) use of a mixture of all solvents at concentrations greater than 500 mg/liter for each substrate as a sole carbon and energy source, which permitted development of both the cometabolism and microbial interaction phenomena (3, 14, 15), favoring the adaptation mechanisms (1, 30, 32).

No growth on chlorinated compounds and very little growth on nonchlorinated compounds were detected in the monophasic aqueous system (Fig. 1) during the acclimation period. However, in the biphasic system, acclimation periods were clearly defined by both a progressive increase in microbial activity (growth rate) and a progressive decrease in lag periods (Fig. 1 through 3). After the acclimation periods stable activity was observed in the adapted consortia. It is evident from these results that the microbial selection process was more efficient in the biphasic system than in the monophasic system, although the same activated sludge was used as the microbial source. Tables 2 and 3 show the growth rate of each strain on each substrate. Although many strains in pure culture do not degrade certain compounds, as is the case for *Alcaligenes* sp. on 1,2,3-trichlorobenzene, *Micrococcus* sp. and *Candida* sp. on butyraldehyde, *Pseudomonas* sp. strain Ps2 on ethyl butyrate and 2-ethylbutyraldehyde, and *Acetobacterium* sp. on 2-ethylbutyraldehyde and butyraldehyde, the consortium was capable of degrading all of the substrate mixtures. Certain "artificial pathways" were probably formed in the microbial communities by the concerted action of popula-

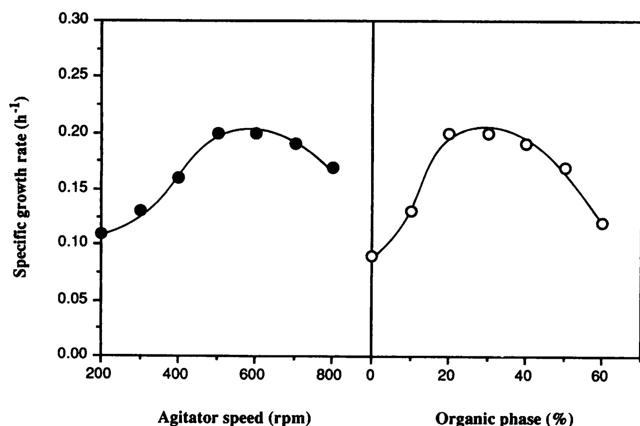

FIG. 4. Variation in the specific growth rate of *Candida* sp. cultivated on 2-ethylbutyraldehyde (3 g/liter) as a function of different silicone oil concentrations and agitation rates. Cultures were incubated at 25°C and pH 4.5 with initial concentrations of dissolved oxygen equivalent to approximately 65% oxygen saturation.

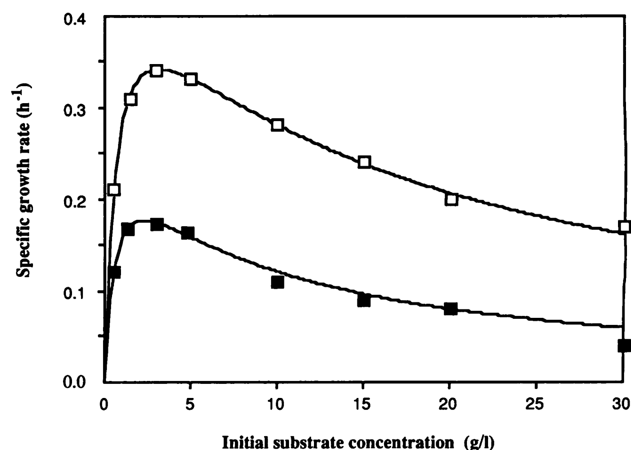

FIG. 5. Specific growth rate of isolated consortium MC2-b as a function of different concentrations of nonchlorinated mixed compounds (concentrations ranged from 0.5 to 30 g/liter, with the same proportion of each substrate), determined for both a monophasic aqueous system (■) and a biphasic aqueous-organic system containing 20% silicone oil (□). Cultures were incubated at 25°C, pH 4.5, and 500 rpm.

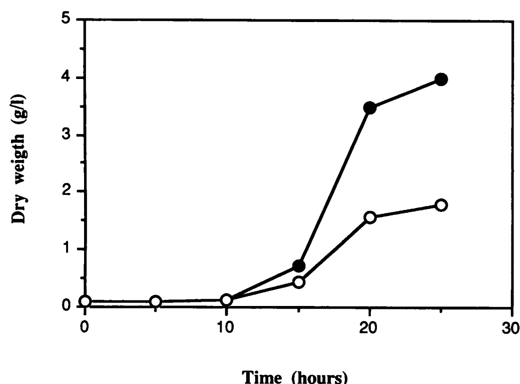

FIG. 6. Partitioning of *Trichosporon* sp. cells between the aqueous and silicone oil phases during ethyl butyrate degradation after agitation was stopped. Cultures were incubated at 25°C, pH 4.5, and 500 rpm in the presence of 20% silicone oil with 4 g of substrate per liter. Symbols: ○, biomass on the water-silicone oil interface; ●, total biomass.

tions during the acclimation period (3, 10). This means that the metabolic sequence of each population contributed to total xenobiotic compound degradation. A similar process occurs in nature during the natural selection of mixed populations on mixed substrates.

The variation in growth rate as a function of the degree of dispersion, as determined by using different silicone oil concentrations and agitation rates with similar initial concentrations of dissolved oxygen for all treatments (Fig. 4), reveals the important role played by the interfacial area in substrate transport between phases, as well as substrate transformation (5, 12, 15, 22). Therefore, the higher growth rate (0.20 h^{-1}) could have been achieved at an optimal interfacial area, which in our case corresponded to silicone oil concentrations between 20 and 40% and to agitation rates between 400 and 700 rpm. In contrast, the low growth rates obtained below or above these organic phase concentration and agitation rate values could have been limited by both the substrate transfer rate and the excess substrate concentration. Similar tendencies were reported by Harbron et al. (12) and Harrop et al. (13) during cultivation of *P. putida* on 1,7-octadiene and naphthalene in biphasic aqueous-organic systems.

Evidence that microbial growth could have occurred both in the aqueous phase and at the liquid-liquid interface is shown in Fig. 6. It was demonstrated that when the agitation was stopped, 45% of the total biomass was bound to the interface. Although the measurements were determined in a nonagitated state, we assume that microbial adhesion also existed during agitated culture growth. Microscope observations (unpublished data) revealed cells covering the silicone oil drops. Microbial adhesion may have been favored by both the silicone oil and cellular hydrophobicity. This phenomenon is favored in the presence of hydrophobic solvents (26) and by changes in cell hydrophobicity during the cellular cycle (2). However, as has been observed in previous experiments, the fractions of biomass at the interface may vary as a function of the organic phase/aqueous phase ratio and substrate concentration (20).

It has been reported that the silicone oils can be degraded slowly and at very low concentrations (100 to 600 mg/liter) by some *Pseudomonas* species (31). However, the high level of performance of the biphasic water-silicone oil system in the degradation of xenobiotic compounds compared with the monophasic aqueous system (Fig. 5) could not be attributed to the utilization of silicone oil as a substrate, since in our case this solvent proved to be inert to microbial attack at the high concentrations used. As shown in Fig. 2, no microbial growth was observed on silicone oil during the enrichment process. Therefore, the difference between these two systems can be explained by the partitioning effect on substrate concentration of the silicone oil during the degradation process. This solvent probably facilitated a shift in reaction equilibria by supplying substrates and extracting products between the phases (28) resulting in a decrease in the inhibition effects of these compounds (12, 17, 19, 25). The concentrations of xenobiotic compounds in the biphasic aqueous organic system when a 20% organic phase was used (Table 1) led to low and nontoxic concentrations of xenobiotic compounds in the aqueous phase. The substrate inhibition effect (Fig. 5) was lower in the biphasic culture (K_i, 16 g/liter) than in the monophasic system (K_i, 8.5 g/liter). Moreover, strains isolated in the monophasic aqueous system exhibited higher levels of activity in the biphasic aqueous-organic system than in the monophasic system (Table 3). Also, as reported previously (22), addition of silicone oil reduces the evaporation of volatile substrates (unpublished data). Thus, the high levels of microbial activity obtained in the biphasic water-silicone oil system could have been the result of microenvironment optimization.

We have shown that the presence in a culture system of a hydrophobic organic solvent having inert physical and chemical characteristics can both accelerate the adaptation of xenobiotic compound-degrading microorganisms and increase xenobiotic compound degradation. Therefore, the biphasic water-silicone oil system could be useful for both microbial selection and degradation of poorly water-soluble xenobiotic compounds. Batch or continuous culture techniques can be used (9, 20, 23, 33), and it is possible that other solvents could be used as the organic phase (19, 20, 22, 25) in these processes.

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